

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Amagai et al.)
)
U. S. Serial No: 09/937,739)
)
Filed: September 28, 2001)
)
PCT International)
Application No: PCT/JP00/02023)
)
Filed: 30 March 2000)
)
Claiming Priority from)
Japanese Application No: 11/91408)
)
Priority Date: 31 March 1999)
)
For : AUTOIMMUNE DISEASE MODEL ANIMAL)
)

STATEMENT IN ACCORDANCE WITH 37 C.F.R. §§ 1.821(f) and (g)

Commissioner for Patents
Washington, D.C. 20231
Box: PCT

Sir:

In accordance with 37 C.F.R. §§ 1.821(f) and (g), this statement confirms that the contents of the Sequence Listing (1 page) of the subject application as filed on September 28, 2001 and on the computer readable 3.5" Diskette submitted herewith are the same and include no new matter.

Respectfully submitted,

Date: March 5, 2002

Georgia Evans
Georgia Evans
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Certificate of Mailing - 37 CFR 1.8(a)	
I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231, on the date below.	
Date <u>3/5/02</u>	<u>Peggy Dirmeyer</u> Peggy Dirmeyer

COMBINED DECLARATION FOR PATENT
APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER
201487/1070 (KUV-101PCT-US)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

AUTOIMMUNE DISEASE MODEL ANIMAL

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as U.S. Patent Application Serial No. _____ on _____ and was amended on _____ (if applicable).

☒ was filed as PCT International Application No. **PCT/JP00/02023** on **March 30, 2000** and assigned U.S. Patent Application Serial No. **09/937,739**.

I hereby state that I have reviewed and understand the contents of the above-identified specifications, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim priority benefits under Title 35, United States Code, § 119 of any application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States listed below and have also identified below any application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (IF PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
Japan	11/91408	31-MARCH-1999	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT International filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS		STATUS (Check One)			
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED	
PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)			
PCT/JP00/02023	30-MARCH-2000	09/937,739		X	

FORM PTO-1390 (Modified)
(REV 11-98)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES

201487/1070

DESIGNATED/ELECTED OFFICE (DO/EO/US)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

CONCERNING A FILING UNDER 35 U.S.C. 371

09/937739

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/JP00/02023

30 March 2000

31 March 1999

TITLE OF INVENTION

AUTOIMMUNE DISEASE MODEL ANIMAL

APPLICANT(S) FOR DO/EO/US

AMAGAI, Masayuki; NISHIKAWA, Takeji; SUZUKI, Harumi; and KOYASU, Shigeo

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

UNSIGNED Combined Declaration and Power of Attorney.
 Diskette containing sequence listing.
 Verified statement re sequence listing.

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.53) 09/937739	INTERNATIONAL APPLICATION NO. PCT/JP00/02023	ATTORNEY'S DOCKET NUMBER 201487/1070
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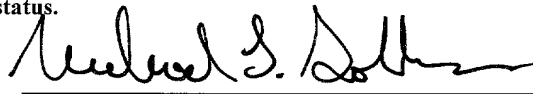
21. The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :				CALCULATIONS PTO USE ONLY	
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1,000.00					
<input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00					
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00					
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00					
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (c)).				\$130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	24 - 20 =	4	x \$18.00	\$72.00	
Independent claims	3 - 3 =	0	x \$80.00	\$0.00	
Multiple Dependent Claims (check if applicable). <input type="checkbox"/>				\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$1,062.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). <input type="checkbox"/>				\$0.00	
SUBTOTAL =				\$1,062.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). <input type="checkbox"/>				\$0.00	
TOTAL NATIONAL FEE =				\$1,062.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input type="checkbox"/>				\$0.00	
TOTAL FEES ENCLOSED =				\$1,062.00	
				Amount to be refunded	\$
				charged	\$

- ☒ A check in the amount of **\$1,062.00** to cover the above fees is enclosed.
- ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.
- ☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **14-1138** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Michael L. Goldman
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United States of America


SIGNATURE

Michael L. Goldman

NAME

30,727

REGISTRATION NUMBER

September 28, 2001
DATE

Docket No: 201487/1070 (KUV-101PCT-US)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Amagai et al.)
)
U. S. Serial No: To be assigned)
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Filed: Herewith)
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PCT International)
Application No: PCT/JP00/02023)
)
Filed: 30 March 2000)
)
Claiming Priority from)
Japanese Application No: 11/91408)
)
Priority Date: 31 March 1999)
)
For : AUTOIMMUNE DISEASE MODEL ANIMAL)
)

PRELIMINARY AMENDMENT

Commissioner of Patents
and Trademarks
Washington, D.C. 20231
BOX: PCT

Dear Sir:

Please amend the above-identified patent application as follows:

In the Claims:

Please cancel claim 1 and add new claims 19-25 as follows:

19. The non-human mammal of claim 3, wherein the immune cells are transplanted to an immunodeficient non-human mammal.

20. The non-human mammal of claim 19, wherein the immunodeficient non-human mammal is a non-human mammal that lacks the RAG2 gene.

21. The non-human mammal of claim 3, wherein the immune cells are splenocytes.

22. The non-human mammal of claim 3, wherein the autoimmune disease is pemphigus vulgaris.

23. The non-human mammal of claim 22, wherein the antigen protein is desmoglein 3 protein.

24. The non-human mammal of claim 3, wherein the non-human mammal is a rodent.

25. The non-human mammal of claim 24, wherein the rodent is a mouse.

Please amend claims 2, 3, 4, 6, 7, 9, 14, 15, and 17 as follows:

2. (Amended) A non-human mammal showing a phenotype of autoimmune disease through production of an antibody reacting to an antigen protein for an autoimmune disease or T cell activation, wherein immune cells from a non-human mammal lacking an antigen gene for the autoimmune disease have been transplanted to the non-human mammal.

3. (Amended) A non-human mammal showing a phenotype of autoimmune disease through production of an antibody reacting to an antigen protein for an autoimmune disease or T cell activation, wherein immune cells from a non-human mammal that lacks the antigen gene for the autoimmune disease and that has been immunized with the antigen protein have been transplanted to the non-human mammal.

4. (Amended) The non-human mammal of claim 2, wherein the immune cells are transplanted to an immunodeficient non-human mammal.

6. (Amended) The non-human mammal of claim 2, wherein the immune cells are splenocytes.

7. (Amended) The non-human mammal of claim 2, wherein the autoimmune disease is pemphigus vulgaris.

9. (Amended) The non-human mammal of claim 2, wherein the non-human mammal is a rodent.

14. (Amended) The method of claim 11, wherein the immune cells are splenocytes.

15. (Amended) The method of claim 11, wherein the autoimmune disease is pemphigus vulgaris.

17. (Amended) The method of claim 11, wherein the non-human mammal is a rodent.

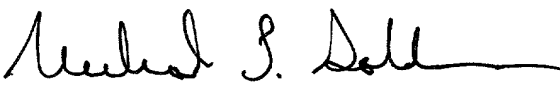
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REMARKS

Pursuant to 37 CFR § 1.121, attached as an Appendix is a version of amended claims 2, 3, 4, 6, 7, 9, 14, 15, and 17 with markings to show changes made.

Respectfully submitted,

Date: September 28, 2001



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001160-664660

Appendix**Version With Markings to Show Changes Made**

In reference to the amendments made herein to claims 2, 3, 4, 6, 7, 9, 14, 15, and 17, additions appear as underlined text, while deletions appear as bracketed text, as indicated below:

In the Claims:

Please amend claims 2, 3, 4, 6, 7, 9, 14, 15, and 17 as follows:

2. (Amended) [The non-human mammal of claim 1] A non-human mammal showing a phenotype of autoimmune disease through production of an antibody reacting to an antigen protein for an autoimmune disease or T cell activation, wherein immune cells from a non-human mammal lacking an antigen gene for the autoimmune disease have been transplanted to the non-human mammal.

3. (Amended) [The non-human mammal of claim 1] A non-human mammal showing a phenotype of autoimmune disease through production of an antibody reacting to an antigen protein for an autoimmune disease or T cell activation, wherein immune cells from a non-human mammal that lacks the antigen gene for the autoimmune disease and that has been immunized with the antigen protein have been transplanted to the non-human mammal.

4. (Amended) The non-human mammal of claim 2 [or 3], wherein the immune cells are transplanted to an immunodeficient non-human mammal.

6. (Amended) The non-human mammal of [any one of claims 2 to 5] claim 2, wherein the immune cells are splenocytes.

7. (Amended) The non-human mammal of [any one of claims 1 to 6] claim 2, wherein the autoimmune disease is pemphigus vulgaris.

9. (Amended) The non-human mammal of [any one of claims 1 to 8] claim 2, wherein the non-human mammal is a rodent.

14. (Amended) The method of [any one of claims 11 to 13] claim 11, wherein the immune cells are splenocytes.

15. (Amended) The method of [any one of claims 11 to 14] claim 11, wherein the autoimmune disease is pemphigus vulgaris.

17. (Amended) The method of [any one of claims 11 to 16] claim 11, wherein the non-human mammal is a rodent.

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DESCRIPTION

AUTOIMMUNE DISEASE MODEL ANIMAL

5 Technical Field

The present invention relates to autoimmune disease model animals and methods for producing them.

Background Art

10 Pemphigus vulgaris (PV) is an autoimmune disease with involvement of skin and mucous membrane blistering, which is sometimes fatal, and is histologically characterized by blistering in the epidermis as well as immunopathologically characterized by the presence of autoantibody IgG to the cell surface of keratinocyte
15 (Stanley, J.R. Pemphigus. In Dermatology in General Medicine. I.M. Freedberg, A.Z. Eisen, K. Wolff, K.F. Austen, L.A. Goldsmith, S.I. Katz, and T.B. Fitzpatrick, eds. McGraw-Hill, New York, 654-666 (1998)). Patients with pemphigus vulgaris clinically manifest diffuse flaccid blister and erosion. These can be formed in all the stratified squamous epithelia. Without appropriate therapy, the widespread lesions on the skin result in the leakage of body fluid or secondary bacterial infection, and as a result pemphigus vulgaris may be fatal. The prognosis of pemphigus can be improved by systemic administration of corticosteroid and immunosuppression therapy, but
20 the mortality remains considerably high because of death due to complications caused by the therapy.

The target antigen for pemphigus vulgaris was first identified as a 130 kD glycoprotein through immunoprecipitation of keratinocyte extract (Stanley, J.R. et al., J. Clin. Invest. 70:281-288 (1982);
30 Stanley, J.R. et al., J. Clin. Invest. 74:313-320 (1984)). Then, cDNA for the pemphigus vulgaris antigen was isolated via immuno-screening of a human keratinocyte expression library using affinity-purified autoantibody specific to the pemphigus vulgaris antigen (Amagai, M. et al., Cell 67:869-877 (1991)). Nucleotide sequence analysis has
35 revealed that the pemphigus vulgaris antigen belongs to the superfamily of genes for cadherins that are intercellular adhesion

molecules. The pemphigus vulgaris antigen is a membrane protein located in desmosome (Karpati, S. et al., J. Cell Biol. 122:409-415 (1993)), and it was named desmoglein 3 (Dsg3) (Amagai, M. Adv. Dermatol. 11:319-352 (1996)).

5 There is much evidence showing that autoantibody IgG against Dsg3 protein plays a pathogenic role in pemphigus vulgaris. Firstly, it has been reported that activity of the disease correlates to the antibody titer in blood over time by indirect fluorescent antibody technique (Sams Jr, W.M. & Jordon, R.E., Br. J. Dermatol. 84:7-13
10 (1971)) or ELISA (Ishii, K., et al., J. Immunol. 159:2010-2017 (1997); Amagai, M., et al., Br. J. Dermatol. 140:351-357 (1999)). Secondly, a newborn from the mother affected with pemphigus vulgaris is also transiently affected with the disease due to the IgG transferred across the placenta from the mother (Merlob, P. et al., Pediatrics 15 78:1102-1105 (1986)). As the IgG derived from the mother is catabolized, the symptom is remitted. Thirdly, the IgG derived from patients with pemphigus vulgaris can induce blistering in tissue-cultured skin in the absence of complement and inflammatory cell (Schultz, J.R., & Michel, B., J. Invest. Dermatol. 67:254-260
20 (1976); Hashimoto, K. et al., J. Exp. Med. 157:259-272 (1983)). Fourthly, passive transfer of the IgG derived from sera of patients into newborn mice causes intraepidermal blister formation with typical histological characteristics (Anhalt, G.J. et al., N. Engl. J. Med. 306:1189-1196 (1982)). Fifthly, depletion of
25 patient-derived serum by immuno-absorption using recombinant Dsg3 protein (rDsg3) comprising extracellular domain thereof removes pathogenicity of the serum and inhibits blistering in newborn mice (Amagai, M. et al., J. Clin. Invest. 94:59-67 (1994)). Finally, antibody affinity-purified with rDsg3 exhibits pathogenicity and thus
30 results in the formation of blister with histological characteristics of pemphigus vulgaris in newborn mice (Amagai, M. et al., J. Clin. Invest. 90:919-926 (1992); Amagai, M. et al., J. Clin. Invest. 102:775-782 (1998)).

35 Based on these studies, pemphigus vulgaris is one of the best-characterized autoimmune diseases with respect to the processes after the generation of autoantibody in particular. Thus pemphigus

vulgaris is now an excellent disease model for tissue-specific autoimmune diseases to study cellular mechanisms underlying the production of autoantibody or destruction of self-tolerance, as well as to develop therapeutic methods specific to the diseases. As the first step toward the goals, it is demanded to develop active disease animal model for pemphigus vulgaris.

Most of experimental autoimmune disease animal models are provided by repeated injection of autoantigen with a variety of adjuvants. However, as exemplified by the case of myasthenia gravis, in which the frequency of generation of the active disease in mice immunized with acetylcholine receptor (T. californica) varies considerably depending on the strains, the success of this method is thus highly empirical (Berman, P.W. et al., Ann. N.Y. Acad. Sci. 377:237-57 (1981)).

Previously, an *in vivo* experimental model for pemphigus vulgaris was developed by the reconstruction of severe combined immunodeficiency (SCID) in mice using PBMC derived from patients with pemphigus vulgaris (Juhasz, I. et al., J. Clin. Invest. 92:2401-7 (1993)). With this model, lymphocytes from the patients produced circulating autoantibody at a low titer, but it was rare that active intraepidermal blistering with deposition of human IgG was found in mouse skin. When human skin was transplanted on SCID mouse, blisters similar to those in pemphigus vulgaris were recognized on the transplanted skin. However, it cannot be denied that the cause of blister formation in this model is an inflammatory response due to the tissue incompatibility with human PBMC and skin. Thus there was no established active disease model for pemphigus vulgaris.

Disclosure of the Invention

The present invention provides autoimmune disease model animals and a method for producing them. More specifically, the present invention provides non-human mammals showing phenotypes of the autoimmune disease in which activation of T cells and B cells reactive to the antigen protein for the autoimmune disease followed by stable production of autoantibody are induced and provides a method for producing them. In a preferable embodiment, the model animal can be

provided by the transplantation of immune cells including B cells producing antibody against the antigen protein of the autoimmune disease and/or T cells that are reactive to the antigen protein.

To achieve the above described objective, first, the present inventors aimed at the production of autoantibody in mice by employing the previously used typical method with repeated injection. Specifically, three strains of mice, BALB/c (H-2^d), C3H/HeJ (H-2^k), and C57BL/6N (H-2^b) were immunized with human or mouse Dsg3 protein. Complete Freund's adjuvant was used in the primary immunization, and then booster immunization was carried out 3 or 7 times by using incomplete Freund's adjuvant. However, with this method, no mice produced antibody capable of reacting to mouse Dsg3 protein (Table 1) and showed phenotype of pemphigus vulgaris at all.

Based on this result, the present inventors set up the hypothesis that self-tolerance to Dsg3 protein prevents the production of pathogenic antibody in mouse body. According to the hypothesis, it can be assumed that the immune system is not exposed to Dsg3 protein during the developmental stages in Dsg3-deficient mouse created by gene-targeting technique and thus the mouse does not acquire self-tolerance to Dsg3 protein.

In order to demonstrate the hypothesis, the present inventors studied whether it was possible for Dsg3-deficient mouse immunized with Dsg3 protein to produce antibody against Dsg3 protein. From the result, it was revealed that when immunized with Dsg3 protein a homozygous DSG3 gene-deficient DSG3^{-/-} mouse much more efficiently produced the antibody than a heterozygous DSG3 gene-deficient DSG3^{+/-} mouse (Figure 1A). In addition, the antibody produced by DSG3^{-/-} mouse was capable of binding to mouse Dsg3 protein on the keratinocyte, but the antibody from DSG3^{+/-} mouse was not (Figure 1B). Specifically, it was revealed that self-tolerance to DSG3 protein had not been established in DSG3^{-/-} mouse and the produced antibody recognized mouse Dsg3 protein as an antigen.

Thus, the present inventors next aimed at the production of antibody against Dsg3 protein and expression of phenotype of pemphigus vulgaris in RAG2^{-/-} immunodeficiency mouse by collecting splenocytes (which have capability of producing antibody against DSG3 protein)

from DSG3-/- mouse immunized with Dsg3 protein and adoptively transferring them into the immunodeficiency mouse. Such RAG2-/- mice express Dsg3 protein, but the mice have neither mature T cells nor B cells because they are deficient in rearrangement of T cell receptor genes and immunoglobulin genes (namely, they are immunodeficient).

As a result, in RAG2-/- mice in which splenocytes from DSG3-/- mouse had been transplanted, the encounter of Dsg3 protein-specific lymphocytes among splenocytes with endogenous Dsg3 protein resulted in permanent production of the antibody against Dsg3 protein (Figure 2A). In addition, it was found that RAG2-/- mice having the immunized DSG3-/- splenocytes showed nearly identical phenotype of DSG3-/- mouse (Koch, P.J., et al., J. Cell Sci. 111:2529-2537 (1998); Koch, P.J., et al., J. Cell Biol. 137:1091-1102 (1997)). All of the mice exhibited erosive lesions in mucous membranes with epidermal separation just above the basal cell layer and telogen hair loss (Figure 3). The presence of nearly identical phenotype reproduced by adoptive transfer of DSG3-/- splenocytes in RAG2-/- recipient mice demonstrated that the produced antibody was specific and pathogenic.

The specificity of the antibody can also be verified by the fact that the *in vivo* deposition is not detectable in other simple epithelia expressing Dsg2 protein (Schafer, S. et al., Exp. Cell Res. 211:391-9 (1994)) and upper part of epidermis expressing Dsg1 protein (Figure 3G) (Amagai, M. et al., J. Invest. Dermatol. 106:351-355 (1996)).

Thus, the present invention provides the first disease mouse model for pemphigus and a method for producing them. The method of the present invention, because of the nature thereof, can be widely applicable to the preparation of model animals for other autoimmune diseases in which associated autoimmune targets have been identified.

Accordingly the present invention relates to autoimmune disease model animals and a method for producing them, more specifically relates to:

(1) a non-human mammal showing a phenotype of autoimmune disease through production of an antibody reacting to an antigen protein for an autoimmune disease or T cell activation;

(2) the non-human mammal of (1), wherein immune cells from a non-human mammal lacking an antigen gene for the autoimmune disease have been

transplanted to the non-human mammal;

(3) the non-human mammal of (1), wherein immune cells from a non-human mammal that lacks the antigen gene for the autoimmune disease and that has been immunized with the antigen protein have been

5 transplanted to the non-human mammal;

(4) the non-human mammal of (2) or (3), wherein the immune cells are transplanted to an immunodeficient non-human mammal;

(5) the non-human mammal of (4), wherein the immunodeficient non-human mammal is a non-human mammal that lacks the RAG2 gene;

10 (6) the non-human mammal of any one of (2) to (5), wherein the immune cells are splenocytes;

(7) the non-human mammal of any one of (1) to (6), wherein the autoimmune disease is pemphigus vulgaris;

15 (8) the non-human mammal of (7), wherein the antigen protein is desmoglein 3 protein;

(9) the non-human mammal of any one of (1) to (8), wherein the non-human mammal is a rodent;

(10) the non-human mammal of (9), wherein the rodent is a mouse;

20 (11) a method for producing a non-human mammal showing a phenotype of autoimmune disease through production of an antibody reacting to an antigen protein for an autoimmune disease or T cell activation, which comprises the steps of:

(a) immunizing, with the antigen protein for the autoimmune disease, a non-human mammal that lacks the antigen gene for the autoimmune disease,

(b) preparing immune cells from the non-human mammal, and

(c) transplanting the immune cells to a non-human mammal having the antigen protein;

30 (12) the method of (11), wherein the immune cells are transplanted to an immunodeficient non-human mammal;

(13) the method of (12), wherein the immunodeficient non-human mammal is a non-human mammal that lacks the RAG2 gene;

(14) the method of any one of (11) to (13), wherein the immune cells are splenocytes;

35 (15) the method of any one of (11) to (14), wherein the autoimmune disease is pemphigus vulgaris;

(16) the method of (15), wherein the antigen protein is desmoglein 3 protein;

(17) the method of any one of (11) to (16), wherein the non-human mammal is a rodent; and

5 (18) the method of (17), wherein the rodent is a mouse.

The model animal of the present invention can show phenotype of autoimmune disease through the stable production of antibody reacting to the antigen protein for the autoimmune disease or sustained activation of T cell.

10 There is no particular restriction on the type of objective disease for which model animals are to be prepared in accordance with the present invention, as far as the disease is an autoimmune disease. Such autoimmune diseases include, for example, but not limited to, pemphigus vulgaris, myasthenia gravis, autoimmune hemolytic anemia, 15 Basedow's disease, Hashimoto's disease, Goodpasture's syndrome, autoimmune diabetes mellitus, multiple sclerosis, etc.

Animals to be utilized for creating the model animal are preferably non-human mammals. There is no restriction on such non-human mammals, as far as gene-disrupted animals can be created 20 from them. Preferable animals include rodents, e.g., mouse.

The model animals in accordance with the present invention can be created by immunizing antigen gene-deficient non-human mammals with the antigen protein for the autoimmune disease, removing the immune cells thereof, and then transplanting the cells to other 25 non-human mammals having the antigen protein.

Animals having the disputed antigen gene can be created by a method known to those skilled in the art. The antigen gene to be disrupted includes, for example, but not limited to, the DSG3 gene when the autoimmune disease is pemphigus vulgaris; the acetylcholine 30 receptor gene for myasthenia gravis; the TSH receptor gene for Basedow's disease or Hashimoto's disease; the type IV collagen gene for Goodpasture's syndrome; the myelin basic protein gene for multiple sclerosis, etc.

Further, immune cells can be obtained from the thymus, lymph 35 node, spleen, liver, intestinal epithelium, peripheral blood, etc. but are not limited to those from the tissues. The spleen abundantly

contains mature immune cells and thus is a preferable organ for the immune cells. It is preferable that the animal (donor) from which immune cells are prepared and the animal (recipient) to which lymphocytes derived from the immune cells are transferred belong to a same species and have a same genetic background thereby preventing the onset of GVHD which may cause tissue destruction in the recipient.

In addition to this, it is preferable that the recipient has immunodeficiency thereby preventing the rejection of lymphocytes derived from immune cells transferred. For example, SCID mouse, nude mouse as well as an animal of which RAG2 gene has been disrupted may be used as the immunodeficient animal. Furthermore, MHC-knockout mouse or common γ chain-knockout mouse can also be used but it is not limited thereto.

The immunization with the antigen protein from the donor, preparation of immune cells from the donor, and transplantation of the immune cells to the recipient can be carried out, for example, by the methods as described in the Examples.

The model animal created in accordance with the present invention can show phenotype of autoimmune disease through the stable production of antibody reacting to the antigen protein for the autoimmune disease or sustained activation of T cell. In the model animal for pemphigus vulgaris, major phenotype includes weight loss and reversible telogen hair loss. Further, among autoimmune diseases other than pemphigus vulgaris, phenotype may include reduced muscle power in myasthenia gravis; anemia in autoimmune hemolytic anemia; hyperthyroidism in Basedow's disease; hypothyroidism in Hashimoto's disease; nephropathy and pulmonary disorders in Goodpasture's syndrome; glucosuria in autoimmune diabetes mellitus; and neuroparalysis in multiple sclerosis.

One can use these model animals for developing therapeutic agents or methods for the diseases, administering to them test compounds of interest for therapeutic effects on autoimmune diseases and observing phenotypes thereof. Particularly the major phenotype includes weight loss and reversible telogen hair loss in pemphigus vulgaris model mouse prepared in accordance with the present Example and the phenotypes last over 6 months, and therefore it is possible

to readily and objectively evaluate the effectiveness of each therapeutic agent or method based on the observation without sacrificing the mouse. In addition, these model mice are very useful for clarifying cellular mechanism underlying the production of antibody against the antigen protein.

Brief Description of the Drawings

Figure 1 shows a diagram (A) and photograph (B) displaying the production of anti-Dsg3 IgG that is capable of *in vivo* binding in DSG3-/- mouse. (A): DSG3-/- mouse and the +/- littermate mice thereof were immunized with mouse rDsg3, and then the titers of anti-Dsg3 IgG were measured over time by ELISA. An arrow indicates the primary immunization with mouse rDsg3 using complete Freund's adjuvant, and an arrow head indicates booster immunization with mouse rDsg3 using incomplete Freund's adjuvant. DSG3-/- mouse efficiently produced much more anti-Dsg3 IgG than the +/- littermate mice. (B): while intercellular junctions of cultured keratinocytes were stained with the serum derived from the immunized DSG3-/- mouse (a), they were not stained with the serum derived from the +/- littermate mice (b). Bar represents 50 μ m.

Figure 2 shows the production of anti-Dsg3 IgG in recipient RAG2-/- mice after the transfer of the immunized DSG3-/- splenocytes. (A): ELISA scores to mouse rDsg3 were obtained with RAG2-/- mouse that had received splenocytes from the immunized DSG3-/- mouse [RAG2-/- (DSG3-/-)] or RAG2-/- mouse that had received splenocytes from the immunized DSG3+/- mouse [RAG2-/- (DSG3+/-)]. The RAG2-/- mouse having DSG3-/- splenocytes had the sustained production of anti-Dsg3 IgG. An arrow indicates the first day of telogen hair loss phenotype. In contrast, ELISA for RAG2-/- mouse having DSG3+/- splenocytes was always negative over time. (B): the time course of varying body weight of recipient RAG2-/- mouse was plotted. After 10 to 14 days, the increase in weight delayed in RAG2-/- mice having DSG3-/- splenocytes as compared with mice having DSG3+/- splenocytes, and then the weight continued to decrease. Several mice died (\dagger), but the remaining several mice survived and then their weights increased.

Figure 3 is a photograph showing the expression of pemphigus vulgaris phenotype in RAG2-/- mice in which the immunized DSG3-/- splenocytes have been transferred. Around 7 to 14 days after the transfer of splenocytes, weight loss was recognized in RAG2-/- mice having DSG3-/- splenocytes (A, bottom) when compared with mice having DSG3+/- splenocytes (A, top). Several mice showed the onset of erosion with scab around noses and cheeks where they scratched (B). Based on histological diagnosis of RAG2-/- mouse having DSG3-/- splenocytes, intraepidermal blister formation was found immediately above the basal layer of mucosal epithelium (C, hard palate; D, upper part of the esophagus). Inflammatory infiltrates were recognized below the erosion foci (E, upper part of the esophagus). In vivo IgG deposition was recognized on cellular surface of keratinocyte in mucosal epithelium by direct immunofluorescence method (F, hard palate) and skin (G, around nose) (white part of the diagram). Bar represents 50 μ m.

Figure 4 is a photograph showing a telogen hair loss phenotype of RAG2-/- mouse, in which the immunized DSG3-/- splenocytes had been transferred, similar to that observed in DSG3-/- mouse. About 15 to 25 days after the transfer, RAG2-/- mouse having DSG3-/- splenocytes showed partial hair loss (A, B). In hair-pull test with adhesive tape, a bunch of hairs were adhered on the tape in the case of RAG2-/- mouse having DSG3-/- splenocytes (C, left), but no hair was adhered in the case of RAG2-/- mouse having DSG3+/- splenocytes (C, right). After that, a mosaic of new hair was recovered in the area without hair (D, arrow). Histological diagnosis showed the presence of acantholysis between cells of hair bulb and basal layer of outer root sheath epithelium (E, arrow) as well as the presence of empty expanded telogen hair follicle (F, arrow). By direct immunofluorescence method, in vivo IgG deposition was found on the cellular surface of keratinocyte in the hair root (G, H) (white part of the diagram). Bar represents 50 μ m.

Best Mode for Carrying out the Invention

The present invention is illustrated in detail below with reference to Examples, but is not to be construed as being limited

thereto.

Example 1. Production of recombinant mouse Dsg3 protein

A cDNA encoding the entire extracellular domain of mouse Dsg3 (Genbank U86016) was amplified by PCR using appropriate primers (5'-CCGAGATCTCCTATAAATATGACCTGCCTCTTCCCTAGA-3'/SEQ ID NO: 1, 5'-CGGGTCGACCCCTCCAGGATGACTCCCCATA-3'/SEQ ID NO: 2) and using a phage clone containing mouse Dsg3 cDNA (a gift from Dr. Jouni Uitto) as a template; amplified fragment was subcloned (pEVmod-mDsg3-His) by replacing it with human Dsg3 cDNA in pEVmod-Dsg3-His vector (Ishii, K., et al., J. Immunol. 159:2010-2017 (1997)). A recombinant baculo-protein, mouse rDsg3, was prepared as described previously (Amagai, M. et al., J. Clin. Invest. 94:59-67 (1994); Amagai, M. et al., J. Invest. Dermatol. 104:895-901 (1995)).

Example 2. Immunization of wild-type DSG3+/+ mouse with mouse Dsg3 protein

First, attempts were made to produce antibodies against Dsg3 protein in a variety of wild-type mouse strains after immunizing with human or mouse rDsg3 (Table 1).

Mice were sensitized with 5 µg of purified mouse or human rDsg3 by intraperitoneal injection with complete Freund's adjuvant (CFA). Then booster immunization was carried out every week, 3 or 7 times, with mouse or human rDsg3 using incomplete Freund's adjuvant (IFA). An ELISA test for antibody production was conducted 3 days after each booster immunization.

In ELISA assay for blood IgG against mouse Dsg3 protein (mDsg3) or human Dsg3 protein (hDsg3) in mice, mouse or human rDsg3 was used as a coating antigen. More specifically, a 96-well microtiter plate was coated with 100 µl of 5 µg/ml purified mouse or human rDsg3 at 4°C overnight. All serum samples were diluted 50 to 5,000 times and then incubated on a 96-well ELISA plate at room temperature for 1 hour. After the samples were incubated with peroxidase-conjugated goat anti-mouse IgG antibody (MBL, Nagoya, Japan) at room temperature for 1 hour, the coloring reaction was carried out by using 1 mM tetramethylbenzidine as a substrate for peroxidase (Ishii K et al.,

J Immunol 159: 2010-2017, 1997; Amagai M et al., Br J Dermatol 140:351-357, 1999). The respective samples were analyzed in duplicate. A single serum sample obtained from DSG3-/- mouse immunized with mouse rDsg3 was used as a positive control and serum
 5 derived from a non-immunized mouse was used as a negative control. ELISA score was obtained as an exponent in a value calculated by $[(\text{sample OD} - \text{negative control OD}) / (\text{positive control OD} - \text{negative control OD}) \times 100]$ (Table 1).

Further, the production of antibody against Dsg3 protein was
 10 tested by immuno-fluorescent staining of cultured keratinocytes. Mouse keratinocytes from cell line PAM212 (Yuspa, S.H. et al., Cancer Res. 40:4694-4703 (1980)) or human keratinocytes from cell line KU8 (Tsukamoto, T., Keio J. Med. 38:277-293 (1989)) were incubated together with mouse serum sample 20-fold diluted with DMEM containing
 15 10% FCS at 37°C under humid air containing 5% CO₂ for 30 minutes. Subsequently the cells were washed with PBS(-) and then fixed with 100% methanol at -20°C for 20 minutes; the cells were incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody (DAKO, Copenhagen, Denmark) at room temperature for 30
 20 minutes. The stain was observed with a fluorescence microscope (Nikon, Eclipse E800).

The ELISA and immuno-fluorescence staining revealed that C57BL/6N mouse or BALB/c mouse produced IgG capable of reacting to only human rDsg3 but not to mouse rDsg3 when immunized with human
 25 rDsg3 first using complete Freund's adjuvant and then using incomplete Freund's adjuvant. Merely C3H/HeJ mice produced IgG capable of weakly cross-reacting to mouse rDsg3, when observed by ELISA. When these three strains of mice were immunized with mouse rDsg3, no mice from the three strains produced IgG recognizing mouse or human rDsg3
 30 in the evaluation by three methods (ELISA, indirect fluorescent antibody technique, and living keratinocyte staining). These findings suggested that wild-type DSG3+/+ mouse had immuno-tolerance to Dsg3 and thus it was difficult to allow for DSG3+/+ wild-type mouse to produce antibody against mouse Dsg3.

Table 1

Mouse strain	n	Antigen ^a	CFA	IFA ^b	ELISA ^c		IIF ^d		Living cell staining ^e	
					mDsg3	hDsg3	NMS	NHS	Pam	KU8
C57BL/6N	2	hDsg3	1	3	18.4	93.4	-	+	-	+
BALB/c	3	hDsg3	1	3	6.3	85.4	-	+	-	+
C3H/HeJ	2	hDsg3	1	3	47.5	167.7	-	+	-	+
C57BL/6N	3	mDsg3	1	7	10.6	10.1	-	-	-	-
BALB/c	3	mDsg3	1	7	1.7	2.3	-	-	-	-
C3H/HeJ	3	mDsg3	1	7	5.4	7.5	-	-	ND	ND

When recombinant human Dsg3 was used, "hDsg3" is provided in the column of antigen in Table 1; when recombinant mouse Dsg3 was used, "mDsg3" is provided (a). In the column of "CFA," the number of immunization treatments with purified mouse or human recombinant Dsg3 using complete Freund's adjuvant (CFA) is indicated; in the column of "IFA," the number of booster immunizations conducted every week after the first one using incomplete Freund's adjuvant (IFA) (3 or 7 times) is indicated (b). ELISA score was computed for human recombinant Dsg3 (hDsg3) or mouse recombinant Dsg3 (mDsg3) (c); if higher than 20.0, it can be judged as positive. "IIF" of this Table indicates a result of indirect fluorescence antibody staining (IIF) of normal mouse skin (NMS) or normal human skin (NHS) by using mouse serum (d). Further, "living cell staining" of this Table indicates a result of living cell staining for cultured mouse keratinocytes from a cell line (Pam) or human keratinocytes from a cell line (KU8) by using the mouse serum (e). "-" means negative; "+" means positive. "ND" indicates that the test was not done.

Example 3. Immunization of DSG3-/- mouse and DSG3+/- mouse with mouse Dsg3 protein

DSG3-/- mice were prepared by mating male DSG3-/- mice with female DSG3+/- mice (Koch, P.J., et al., J. Cell Biol. 137:1091-1102 (1997)). RAG2-/- mice, which had been obtained by back-crossing with

B6.SJL-ptpr^c over 10 generations, were provided from Taconic (German Town, NY) (Schulz, R.-J. et al., J. Immunol. 157:4379-4389 (1996)).

ELISA scores for mouse rDsg3 were determined after immunizing DSG3-/- mouse with mouse rDsg3 in order to verify the absence of immuno-tolerance to Dsg3 protein in DSG3-/- mouse.

Both DSG3-/- mice and DSG3+/- mice were sensitized with 5 µg of purified mouse rDsg3 by using complete Freund's adjuvant (0 day), and then booster was carried out with mouse rDsg3 by using incomplete Freund's adjuvant after 8, 15, 22, and 28 days. The antibody production was tested by ELISA using mouse rDsg3 as a coating antigen in the same manner as in Example 2.

The production of anti-Dsg3 IgG was found as early as 11th day in DSG3-/- mice (n=4) and the titer continued to increase (Figure 1A). When DSG3+/- mice were immunized repeatedly, the ELISA titer eventually increased, but the titer was significantly lower than the titer for DSG3-/- mice observed on the 32nd day (p<0.0001).

To determine whether the anti-Dsg3 IgGs produced by these mice can bind to Dsg3 protein on the keratinocytes *in vivo*, the same staining as in Example 2 was carried out by using mouse keratinocytes from a cell line Pam212. When the serum derived from DSG3-/- mouse was added to culture media, the serum bound at intercellular adhesion sites of cultured keratinocytes. However, no stain was detectable on the cell surface by using sera derived from DSG3+/- mouse at all (Figure 1B). There was no *in vivo* IgG deposition in the epidermis of the immunized DSG3+/- mice. Thus there is an extremely high possibility that the antibodies produced by DSG3+/- mouse are those against trace quantities of contaminants in purified mouse rDsg3, against the C-terminal tag of mouse rDsg3, or against masked Dsg3 epitopes which are not accessible under the *in vivo* condition.

These results suggested that there was no immuno-tolerance to Dsg3 in DSG3-/- mouse and thus pathogenic IgG inhibiting Dsg3 function for adhesion was produced via immunizing DSG3-/- mouse with mouse rDsg3.

Example 4. Permanent production of pathogenic anti-Dsg3 IgG in recipient RAG2-/- mouse

Because DSG3^{-/-} mouse has the deficient target antigen, it was predicted that anti-Dsg3 IgG did not affect the phenotype in DSG3^{-/-} mouse. Thus, an experiment was conducted where immunized splenocytes from DSG3^{-/-} mouse or DSG3^{+/-} mouse were transferred into RAG2^{-/-} immunodeficiency mouse. RAG2^{-/-} mouse expresses Dsg3 protein but has neither mature T cells nor B cells because neither T cell receptor genes nor immunoglobulin genes can be rearranged in the mouse. Therefore it is assumed that the transferred splenocytes are not rejected and anti-Dsg3 IgG can be produced in the recipient mouse.

DSG3^{-/-} mice and DSG3^{+/-} mice were sensitized with 5 µg of purified mouse rDsg3 by using complete Freund's adjuvant (0 day). Then booster immunization was carried out with mouse rDsg3 using incomplete Freund's adjuvant after 7 and 14 days. The production of antibody was confirmed on the 18th day by ELISA in the same manner as in Example 2. Finally, booster immunization was carried out with mouse rDsg3 but without any adjuvant, and the mice were sacrificed several days after the booster immunization to prepare splenocytes as immune cells.

To perform the adoptive transfer of splenocytes, monocytes were isolated from the spleens of DSG3^{-/-} mice or DSG3^{+/-} mice and re-suspended in complete RPMI1640 medium (Nissui Pharmaceuticals, Tokyo) containing 10% fetal bovine serum, 0.21% sodium bicarbonate solution (w/v), 2 mM L-glutamine (GIBCO), and antibiotics. About 1×10^7 splenocytes were suspended in PBS and transferred into RAG2^{-/-} mouse via caudal vein by intravenous injection. The production of antibody was tested by ELISA using mouse rDsg3 as a coating antigen in the same manner as in Example 2.

Anti-Dsg3 IgG was detected in the blood of recipient RAG2^{-/-} mice as early as 4th day after the transfer of DSG3^{-/-} splenocytes. The antibody produced rapidly increased and reached a plateau around the 21st day; the production then continued permanently (n=13) (Figure 2A). The sustained antibody production was observed for 6 months or more until the mice died. In contrast, anti-Dsg3 IgG was always undetectable over time in the blood of RAG2^{-/-} mice in which DSG3^{+/-} mouse splenocytes had been transferred (n=5) (Figure 2A).

In order to determine the localization of B cells producing

anti-Dsg3 IgG, ELISPOT assay was conducted as follows. A 96-well microtiter plate of which bottom is made of PVDF (Millipore-Amicon, Beverly, MA) was coated with mouse rDsg3 of 30 µg/ml. The monocytes prepared from reconstructed RAG2-/- mouse peripheral blood, spleen, bone marrow, and lymph node were incubated on the plate at 37°C under humid air containing 5% CO₂ for 4 hours. The IgG bound to the membrane was visualized as a spot by using alkaline phosphatase-conjugated anti-mouse IgG antibody (Zymed Laboratories Inc, San Francisco, CA). The number of spots were counted under a stereoscopic microscope, the frequency of B cells producing anti-mDsg3 IgG was determined as the number per 10⁵ monocytes. All the experiments were performed in triplicate. The number of B cells producing anti-Dsg3 IgG determined by the assay is shown in Table 2.

It was revealed that B cells producing anti-Dsg3 IgG were localized in the spleens and lymph nodes of recipient RAG2-/- mice in the early phase (on the 22nd day) as well as late phase (on the 117th day) after the adoptive transfer (Table 2). In this Table, RAG2-/- mice in which splenocytes from immunized DSG3-/- mouse had been transferred is represented by "+"; the mouse which had no transferred cell is by "-" (a). "Days" represents days from the transfer to the sacrifice (b). The number of B cells producing anti-mDsg3 IgG is indicated as the number per 10⁵ monocytes (c). The frequency of B cells producing anti-Dsg3 IgG in the spleen ranged from 20 to 100 cells per 10⁵ monocytes.

Table 2

Mouse	Transfer ^a	Days ^b	Spleen	Lymph node	Bone marrow	PBMC
RAG#466	-	-	0.0±0.0 ^c	0.0±0.0	0.0±0.0	0.0±0.0
RAG#514	+	22	86.5±29.9	13.5±13.6	0.0±0.0	3.8±5.4
RAG#212	+	33	102.1±14.7	47.8±8.8	0.0±0.0	0.0±0.0
RAG#134	+	117	20.8±5.9	16.5±5.9	2.1±2.9	0.0±0.0
RAG#135	+	117	31.3±8.8	27.1±2.9	0.0±0.0	0.0±0.0

Example 5. RAG2-/- mouse having immunized DSG3-/- splenocytes expressed the phenotype of pemphigus vulgaris

The first recognized symptom in recipient RAG2-/- mouse having immunized DSG3-/- splenocytes was weight loss (n=13) as compared with mouse having DSG3+/- splenocytes (n=5) around 7 to 14 days after the adoptive transfer (Figures 2B and 3A). The weights of these mice then continued to decrease and several of them actually died. The remaining survived mice later began to gain their weights (Figure 2B). The phenotype of weight loss was recognized in all the recipient RAG2-/- mice examined (n=13). Several of the recipient RAG2-/- mice (n=5) had the onset of erosion with scab on the skin around the noses which is a common area of scratch (Figure 3B).

In vivo IgG deposition was found on the cell surface of stratified squamous epithelium keratinocyte including epidermis (Figure 3G; around the nose), mucous membrane of oral cavity (Figure 3F, hard palate), and esophagus mucous membrane in recipient RAG2-/- mouse. In epidermis consisting of several layers of keratinocytes, the presence of IgG deposition was restricted to the lower layers (Figure 3G), while IgG was observed in all the epithelial layers in epithelia of oral cavity and esophagus (Figure 3F). IgG deposition was not detected in any other tissues including heart, lung, liver, kidney, stomach, small intestine, and large intestine in these mice. Histological diagnosis of RAG2-/- mice having immunized DSG3-/- splenocytes showed the presence of intraepithelial cleavage immediately above the basal layer, namely, acantholysis immediately above the basal layer which is a typical characteristic of pemphigus vulgaris, in buccal mucous membrane, hard palate (Figure 3C), the oral and pharyngeal region, and upper part of the esophagus (Figure 3D). The significant inflammatory cell infiltrate was not essentially seen in blistering lesions in the early phase (Figure 3C). Inflammatory infiltrate was chiefly found in old erosion foci (Figure 3E). Irritation and acute inflammation secondarily caused by food were recognized there, which were due to loss of epithelial barrier function. It can be presumed that these damages perhaps reduced ingestion in the mice and resulted in growth inhibition.

In contrast, no phenotypic or pathological alterations were recognized in RAG2-/- mice having immunized DSG3+/- splenocytes. These findings suggested that RAG2-/- mice having immunized DSG3-/- splenocytes expressed pemphigus vulgaris phenotype.

5

Example 6. RAG2-/- mouse having immunized DSG3-/- splenocytes exhibited the phenotype of telogen hair loss

10 About 15 to 25 days after the adoptive transfer, partial hair loss was recognized in 11 of 13 RAG2-/- mice (Figure 2A, see arrow, Figure 4A, B). Typically, hair loss was initiated as a small spot, and then was expanding gradually during the next 2 to 3 weeks. Hair loss was initiated in the forehead in recipient RAG2-/- mice of 12-week old or less, and it further expanded backward. A mosaic of new hair was recovered in the telogen hair loss spot in several mice, but there were mice in which telogen hair loss spots remained without changing for one month or more or in which telogen hair loss expanded without forming demarcated telogen hair loss spot (Figure 4D). When adhesive tape was adhered to the area adjacent to a telogen hair loss spot and then removed (hair-pull test) (Koch, P.J., et al., J. Cell Sci. 111:2529-2537 (1998)), a bunch of hairs adhered on the tape (Figure 4C). These phenotypes lasted for 6 months or more as far as anti-Dsg3 IgG was present in the blood.

15 The skin biopsy of RAG2-/- recipient mouse revealed intense IgG deposition on the cell surface of keratinocytes around hair bulb (Figures 4G, H). The intensity of IgG binding in hair follicle was much higher than that in epidermis (Figure 4G). Histological diagnosis of the skin showed the presence of acantholysis between cells around hair bulb at the resting stage and the basal layer of outer root sheath (Figures 4E, H; arrow). In telogen hair loss spots, there were empty expanded telogen hair follicles being consistent with telogen effluvium (Figure 4F). There was no evident acantholysis in the surface layer of epidermis without damage. No obvious infiltration of inflammatory cells was recognized around the hair follicles with acantholysis (Figures 4E, F).

20 In contrast, no telogen hair loss spots were always found in RAG2-/- mice in which DSG3+/- splenocytes had been transferred.

35

Industrial Applicability

5 The development of this model provided a new direction for the study of tissue-specific autoimmune diseases (autoimmune diseases in which the relation between target antigen and toxic antibody or T cell has been clarified). The model of the present invention is useful to elucidate cellular mechanisms underlying the production of antibody against antigen protein for an autoimmune disease and induction of cytotoxic T cell by particularly modifying lymphocytes before adoptive transfer. This model can also be a valuable tool for the development of new disease-specific therapies. Because, in the pemphigus vulgaris model animal in accordance with the present invention, the major phenotypes are weight loss and reversible telogen hair loss, activity of the disease can be monitored by observing the mice without sacrificing them. ELISA titer of blood anti-Dsg3 antibody is also an objective index for the disease activity. Further, the phenotype remains expressed for 6 months or more. Thus efficacy of each therapeutic method can readily and objectively be evaluated. More importantly, the method of the present invention is applicable for the development of active disease mouse models for other tissues specific autoimmune diseases in which target antigens have been identified.

CLAIMS

1. A non-human mammal showing a phenotype of autoimmune disease through production of an antibody reacting to an antigen protein for an autoimmune disease or T cell activation.
2. The non-human mammal of claim 1, wherein immune cells from a non-human mammal lacking an antigen gene for the autoimmune disease have been transplanted to the non-human mammal.
3. The non-human mammal of claim 1, wherein immune cells from a non-human mammal that lacks the antigen gene for the autoimmune disease and that has been immunized with the antigen protein have been transplanted to the non-human mammal.
4. The non-human mammal of claim 2 or 3, wherein the immune cells are transplanted to an immunodeficient non-human mammal.
5. The non-human mammal of claim 4, wherein the immunodeficient non-human mammal is a non-human mammal that lacks the RAG2 gene.
6. The non-human mammal of any one of claims 2 to 5, wherein the immune cells are splenocytes.
7. The non-human mammal of any one of claims 1 to 6, wherein the autoimmune disease is pemphigus vulgaris.
8. The non-human mammal of claim 7, wherein the antigen protein is desmoglein 3 protein.
9. The non-human mammal of any one of claims 1 to 8, wherein the non-human mammal is a rodent.
10. The non-human mammal of claim 9, wherein the rodent is a mouse.
11. A method for producing a non-human mammal showing a phenotype of autoimmune disease through production of an antibody reacting to an antigen protein for an autoimmune disease or T cell activation, which comprises the steps of:
- (a) immunizing, with the antigen protein for the autoimmune disease, a non-human mammal that lacks the antigen gene for the autoimmune disease,
- (b) preparing immune cells from the non-human mammal, and
- (c) transplanting the immune cells to a non-human mammal having the antigen protein.
12. The method of claim 11, wherein the immune cells are transplanted

to an immunodeficient non-human mammal.

13. The method of claim 12, wherein the immunodeficient non-human mammal is a non-human mammal that lacks the RAG2 gene.

5 14. The method of any one of claims 11 to 13, wherein the immune cells are splenocytes.

15. The method of any one of claims 11 to 14, wherein the autoimmune disease is pemphigus vulgaris.

16. The method of claim 15, wherein the antigen protein is desmoglein 3 protein.

10 17. The method of any one of claims 11 to 16, wherein the non-human mammal is a rodent.

18. The method of claim 17, wherein the rodent is a mouse.

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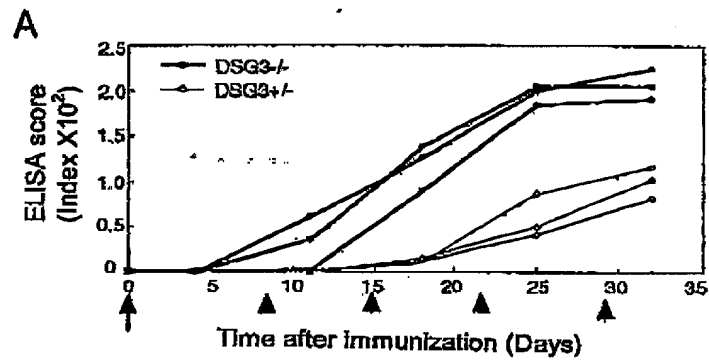
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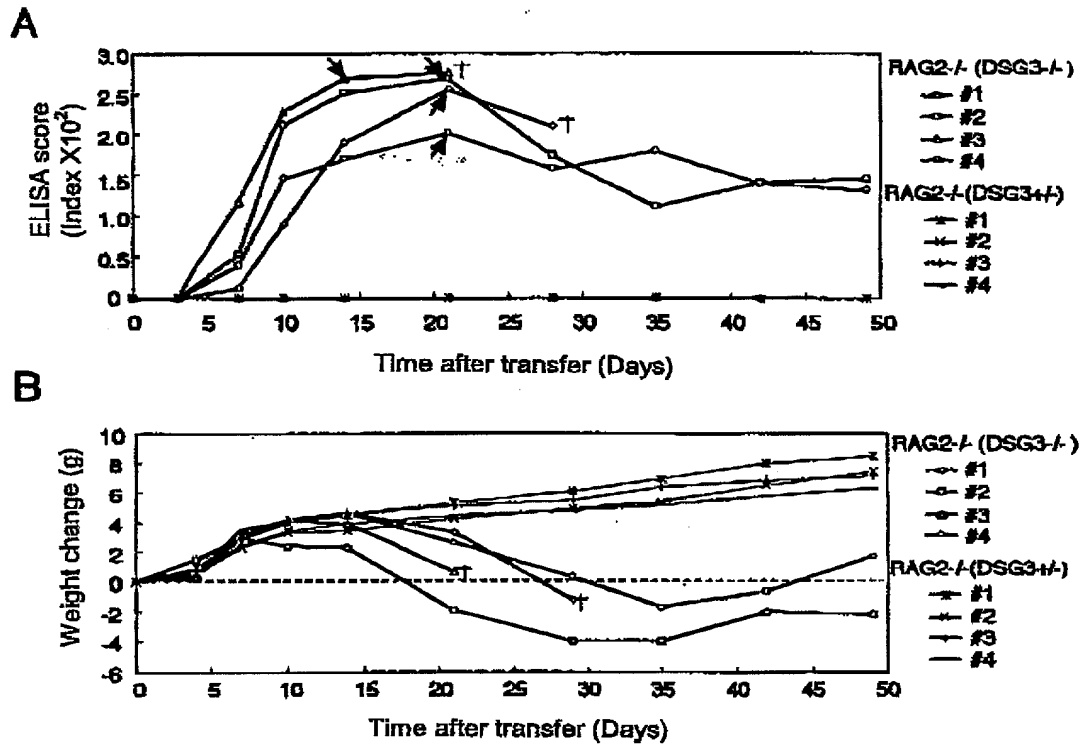
Figure 1

**B**

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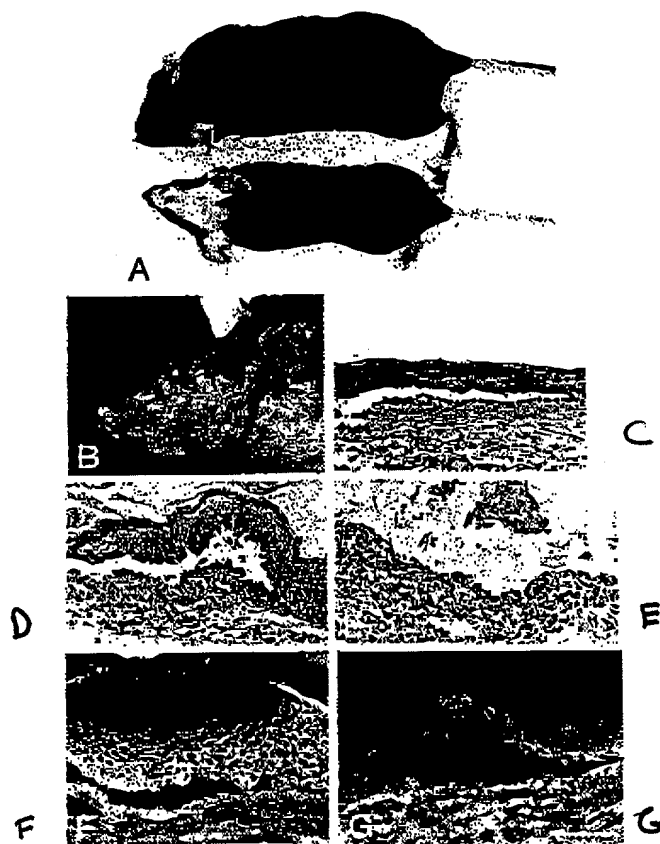
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Figure 2



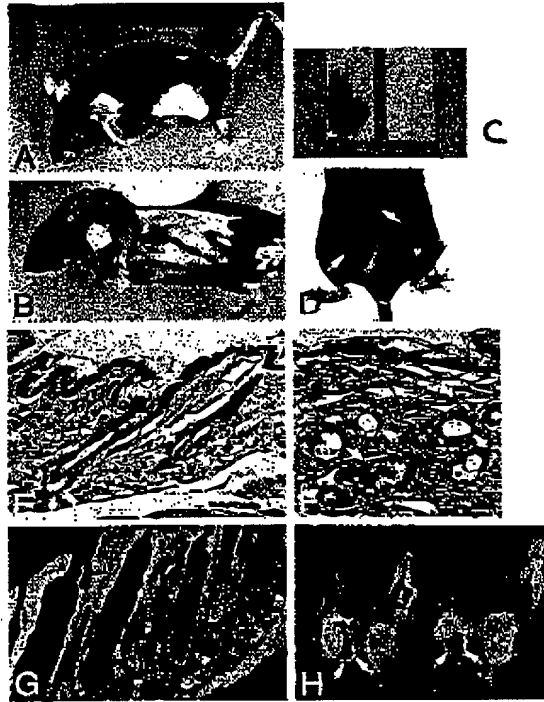
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Figure 3



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Figure 4



I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statement may jeopardize the validity of the application or any patent issuing thereon.

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DATE <i>30th November 2001</i>		

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COMBINED DECLARATION FOR PATENT APPLICATION
AND POWER OF ATTORNEY (Continue)

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SEQUENCE LISTING

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Suzuki, Harumi
Koyasu, Shigeo

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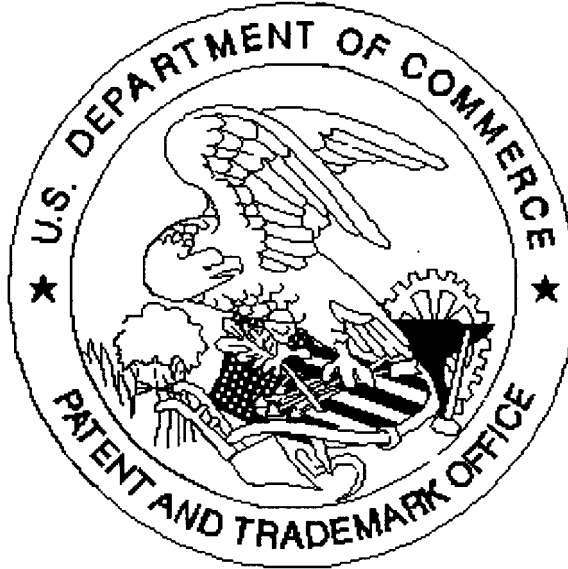
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